Biological warfare between *Drosophila* species hints for sustainable crop protection

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Abstract

Species that share the same ecological niche often avoid costly competition with context-dependent behaviors. This is the case of the invasive insect pest *Drosophila suzukii* that can develop in ripe fruit where it encounters *Drosophila melanogaster*. Previous research showed *D. suzukii* prevents costly larval competition by not ovipositing in substrates with *D. melanogaster* eggs. We discovered *D. suzukii* females respond to cues produced by bacteria in the gut microbiome of *D. melanogaster*. This behavior of *D. suzukii* varied over time and among populations, revealing subtle condition-dependence. In particular, *D. suzukii* females that bore *D. melanogaster* bacteria stopped avoiding sites with *D. melanogaster* cues. The adaptive significance of the behavior was investigated by reproducing experimentally in-fruit larval competition. *D. suzukii* larvae only suffered from competition with *D. melanogaster* if the competitor was associated to its microbiota, suggesting *D. suzukii* has evolved a solution to avoid offspring develops in challenging environments. We argue that *D. suzukii*'s competition avoidance behavior has features enabling the design of an evolution-proof repellent to protect crops.

Introduction

Over the last 10 years, the Asian fly *Drosophila suzukii* (*Ds*) has spread in Europe and the Americas (1) causing important fruit-production losses (2-4). As a consequence, research on this species has multiplied in pest-control and *Drosophila* laboratories. Numerous colleagues readily noticed the contamination of *Ds* cages with *D. melanogaster* (*Dmel*) individuals led to its rapid competitive exclusion (5). This phenomenon can be partly explained by the observation that *Ds* females avoid laying eggs in resource sites that already contain *Dmel* eggs (6). *Ds* females however do not avoid oviposition on sites with conspecifics (Fig. S1), prevention of larval crowding does therefore not explain female oviposition behaviour. We investigated the variability and mechanisms of *Ds* ovipositional response to *Dmel* repellency, its reliance on symbiotic bacteria and possible adaptive origin.

Results and discussion

Variable response of D. suzukii females to D. melanogaster cues

In an early experiment, we followed over four days the egg-laying preferences of *Ds* females that were given the choice to oviposit either on control substrates or substrates previously exposed for 24h to *Dmel* females (Fig. 1). Oviposition substrates were replaced daily. During the first two days, *Ds* females laid the majority of their eggs on sites that had not been exposed to *Dmel*. However, *Ds* females did not show this avoidance during the final two days (Fig. 2a). The experiment shows that *Ds* avoidance behavior is plastic within the lifespan of the adult insect and depends on its experience or physiological condition.

In order to determine how widespread *Ds* avoidance behavior is among fly populations, we tested females from different laboratory populations founded with insects captured in France (i.e. our reference population used throughout this study), the USA, China and Japan (see methods). Flies from most populations exhibited avoidance but not those from Japan (Fig. 2b). *Ds* originates from mainland China, invaded Japan at the beginning of the 20th century and recently Europe and North-America (7). These results hence show that avoidance behavior is neither restricted to invasive populations nor to those from the area of origin.

We further meant to assess whether avoidance behavior was shared between laboratory and wild *Ds* flies. We therefore trapped wild *Ds* adults from the Montpellier, Southern France region, using classical vinegar traps modified to prevent the drowning of captured flies (see methods). These traps attracted various species of Drosophilid flies, among which a number of *Ds* females. Unlike laboratory *Ds* flies, wild *Ds* females did not exhibit avoidance behavior to *Dmel*-exposed substrates (Fig. 2c). We identified three alternative explanations: (1) avoidance behavior is a laboratory artefact ; (2) uncontrolled fly age or pre-capture history affects female selectivity ; or (3) exposure to other Drosophilid flies, including *Dmel*, during the time spent in traps suffices to trigger avoidance loss as we also observed in our first experiment (Fig. 2a).

Our results show that *Ds* oviposition avoidance of sites with *Dmel* cues varies among populations and with individual experience or physiological condition. This contrasts with the sustained and hard-wired oviposition avoidance that *Dmel* females display in response to geosmin (8), a molecule produced by microorganisms responsible for late-stage fruit rot

that are detrimental to *Dmel* larvae. Why and how *Ds* females lose response to *Dmel* cues (Fig. 2a) could be due to, at least, three non-excluding phenomena. First, Drosophila flies have remarkable learning abilities (9) ; *Ds* may therefore learn to tolerate these cues or habituate to them leading to oviposition avoidance loss. Second, females may lose oviposition site selectivity (i.e. take more risks) after 2 days of successful egg-laying. This scenario is unlikely as the *Ds* females used in our experiments had had the opportunity to oviposit before experiment onset. Third, *Ds* exposure to *Dmel* or *Dmel* products (e.g. feces) could modify *Ds* internal state in such way that oviposition avoidance is altered because it is not possible or necessary any more.

Conditions of repellency by *D. melanogaster*

The variable behavioral response of *Ds* prompted us to investigate whether *Dmel*-induced repellency was variable too. As published results and our initial experiments (Fig. 2a and Fig. S1) had been carried out with *Dmel* females or eggs, we next compared the ability of *Dmel* males and females to induce repellency in more detail. The experiment showed that males, like females, induced repellency (Fig. 3a). This rules-out *Dmel* eggs or oviposition associated cues as driving factors, and contrasts with Tephritid fruit-flies that avoid larval crowding using so-called host-marking pheromones (10)

We further investigated possible variations among *Dmel* populations and its sister species. *D. simulans* (*Dsim*), which ecology is very close to that of *Dmel* (11). We tested the repellency of wild *Dmel* trap-captured in Southern France, lab-reared F1 offspring of the same wild

Southern France *Dmel* population, the Oregon-R lab strain of *Dmel* used for all previous experiments. Similar to laboratory flies, wild *Dmel* flies were repellent, however those that had spent one generation in the laboratory were not (Fig. 3b). The *Dsim* population we
135 tested was not repellent. Similarly, fruit exposure to *Ds* did not elicit *Ds* oviposition avoidance (Fig. S1). Repellency is therefore a feature of wild and laboratory *D. melanogaster* populations that is nonetheless sensitive to rearing conditions.

Some of the above results suggest a plausible mechanism behind *Ds* oviposition avoidance
140 and its plasticity. We observed a decrease of *Ds* avoidance behavior after 2 days of exposure to *Dmel* associated products (Fig. 2a) and that both males and females of *Dmel* produced the repellent (Fig. 3a). Besides, we carried-out an additional experiment testing whether repellency restricted to *Dmel*-exposed substrates, or also protected surrounding sites. Lack of effect on substrates neighboring *Dmel*-exposed medium (Fig. S2) was reminiscent of the
145 local effect of *Dmel* gut bacteria inoculated to nutritive media on *Dmel* adult foraging preferences (12, 13). It was shown indeed *Drosophila* foraging preferences depend on the combination of the bacterial species inoculated to the nutritive medium and that symbiotically present in the fly (12). Drosophilids hence possess the sensory and neuronal circuitry to perceive specific bacteria (or cues associated with them) and respond to their
150 presence in function of their own symbionts. It was plausible to suspect a similar ability in *Ds* and hypothesized that microbial symbionts of *Dmel* can be perceived by *Ds* females, and that oviposition avoidance, or its lack, would be a function of *Ds* symbiotic status. We describe below a series of experiment testing this hypothesis.

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Bacterial symbionts of *Dmel* are involved in repellency and *Ds* avoidance loss

In order to investigate the implication of extra-cellular microorganisms in *Dmel* repellency,
160 we tested whether axenic *Dmel* flies were repellent as conventional flies. Because we suspected *Ds* symbiotic status being involved in the loss of oviposition avoidance, we also compared the response of axenic and conventional *Ds* females. Axenic *Dmel* flies did not elicit significant oviposition avoidance in *Ds* (Fig. 4a). Extra-cellular micro-organisms thus participate to *Dmel* repellency. However, both axenic and conventional *Ds* responded to the
165 repellency provoked by conventional *Dmel* (Fig. 4a). It rules out extra-cellular micro-organisms as a necessary condition to perceive repellent cues.

We then tried to restore experimentally repellence in *Dmel* by inoculating axenic flies with candidate bacteria (i.e. creating gnotobiotic flies). The bacterial microbiota of *Dmel* has been
170 extensively described over the last ten years, showing it largely varies among populations and environmental conditions and often comprises species of the genera *Lactobacillus* and *Acetobacter* (14-17). We therefore elected to associate *Dmel* flies with a strain of *Lactobacillus brevis,* or with one of *Acetobacter pomorum*. Both these bacterial strains had been isolated from a laboratory population of *Dmel* and are frequently used for microbiota
175 studies (18, 19). In order to investigate whether any type of bacterium could restore repellency in axenic *Dmel*, we also associated *Dmel* flies with a strain of *Escherichia coli* previously shown as non-pathogenic to flies (20). The two bacterial isolates from *Dmel*, *A. pomorum* and *L. brevis*, enabled *Dmel* repellency while *E. coli* did not (Fig. 4b). This experiment confirms the role of *Dmel* symbiotic association in repellency and shows its

180 bacterial origin. It further implies all bacteria cannot elicit repellency, suggesting a form of specificity in the cues produced by *Dmel* and perceived by *Ds*. The bacterial microbiota of adult *Dmel* flies varies largely among populations and individuals (14-17). These variations likely explain the differences in terms of repellency intensity we observed in the different experiments we conducted. For example, the absence of repellency by F1 offspring from
185 wild *Dmel* may be due to the alteration of their microbiota during the first stages of domestication (21). However, the range of microorganisms able to render *Dmel* repellent cannot be determined on the basis of our study. Further work with wild microorganisms, flies and conditions will be necessary to assess the field prevalence of the symbiont-based phenomenon we unveiled here.

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Why did *Ds* females lose avoidance behaviour following exposure to *Dmel* cues (Fig. 2a)? We hypothesized the decrease of oviposition avoidance was due to colonisation of *Ds* by the microorganisms *Dmel* deposited on oviposition sites. In order to test this hypothesis, we mono-associated adult *Ds* for 5 days with the strain of *L. brevis* that elicited strong
195 repellence by *Dmel* (Fig 4b). As expected, *Ds* females associated with *L. brevis* did not avoid oviposition on substrate that had been exposed to *Dmel* adults bearing the same bacterium (Fig 4c). *Ds* females hence avoided sites with cues indicative of presence of *Dmel* unless they carried similar bacteria. Such plastic loss of avoidance behaviour would make sense, in evolutionary terms, if the bacteria were detrimental to *Ds* larvae and that they were
200 transmitted vertically (or pseudo-vertically) from mothers to offspring (22-24). Contaminated mothers would not avoid poor quality oviposition sites as their offspring would anyways inherit their costly bacteria. Alternatively, avoidance loss could also be due to host manipulation by gut bacteria (25): as *Drosophila* larvae favour the development of

the microorganisms they share fruit flesh with (26), a bacterium in *Ds* mothers could
205 manipulate its host in order to increase larval presence in sites where it is detected. Avoidance loss by bacterium-associated *Ds* females probably explains why trap-captured wild *Ds* females did not avoid *Dmel* cues (Fig. 2c). In the traps, wild *Ds* were in close contact with other Drosophilids from which they likely acquired a diversity of microorganisms.

210 To investigate the possibility of transferring our results to application in pest management, we investigated whether bacteria deposited by *Dmel* were sufficient to repel *Ds* oviposition even in absence of *Dmel* individuals, or if *Ds* flies perceive cues produced by the interaction between *Dmel* and its symbionts. To this end we carried out two experiments. In the first, we tested the repellence of medium inoculated with 1,000,000 bacterial cells. In the second,
215 we inoculated the medium with only 5,000 cells corresponding to the numeration of live bacteria on substrates exposed to *Dmel* in the conditions of our experiments. *Ds* females avoided oviposition on media inoculated with numerous bacterial cells. However, lower numbers of live bacterial missed to elicit avoidance behaviour (Fig. 4d), ruling out bacterial growth on medium as the sole source of repellence. Together, these two experiments
220 suggest that when *Dmel* adults are associated with bacteria, some compounds are produced - by one or the other partner - and later perceived by *Ds* females. Prospects for crop protection will necessitate identifying these compounds and testing them as repellents.

225 **_Ds_ larvae suffer from competition with symbiont-associated _Dmel_ larvae**

Avoidance behavior by *Ds* females could be an adaptation meant to ensure offspring do not develop in poor quality sites. In order to test whether *Ds* larvae suffer from competition with *Dmel* larvae, we mimicked in-fruit competition between the two species. Surface-sterilized grape berries were pierced with a fine-needle and single *Ds* eggs deposited in each hole like *Ds* females do (Fig. S3). Some holes also received axenic or conventional *Dmel* eggs. We deposited either 1 or 5 *Dmel* eggs per *Ds* egg, the greater number was inspired by infestation intensities observed in fruit collected in the field (27). *Ds* developmental success (i.e. proportion of eggs that reached adulthood) was impaired by competition with *Dmel* larvae that were associated with their microbiota, but not with axenic *Dmel* larvae (Fig. 5). In the wild, *Dmel* eggs are never axenic, the normal outcome of larval competition is therefore poor *Ds* development. Results hence support our hypothesis that *Ds* oviposition behavior prevents costly larval competition with *Dmel*. It is noteworthy that unlike in (28), the presence of *Dmel* bacteria (in absence of *Dmel* larvae) - which we obtained by exposing fruit to male *Dmel* - was not detrimental to *Ds* larval development. This illustrates the environmental dependence of bacterial effects onto *Drosophila* phenotype (e.g. 29).

It Is remarkable that axenic *Dmel* larvae failed to reduce *Ds* larval development and probably explains why *Ds* females did not respond to cues produced by axenic *Dmel*. *Ds* females would only avoid oviposition in microbial contexts that are detrimental to their offspring. Bacterial microbiota composition, and its effects on host phenotype, vary widely among *Drosophila* species, populations and individuals (17, 28). The plastic decision by Ds to oviposit, or not, in function of microbiota composition may therefore enable using all suitable oviposition sites, and only avoid sites contaminated with costly competitors when necessary.

Prospect for an evolution-proof repellent to protect crops

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Our study shows the competition between insect species with overlapping ecological niches at the larval stage can be determined by microbial symbionts. Symbionts are further used by egg-laying *Ds* females to choose adequate oviposition sites so as to alleviate competition costs. It is well established that microorganisms impact the outcome of competitive
260 interactions between hosts (30). Often, parasitic microorganisms shed by tolerant species have detrimental effects on less-tolerant competitors (e.g. 31); the spill-over hypothesis that facilitates the spread of some invasive species is based on this very mechanism (30). Symbiotic microorganisms can also elicit beneficial effects for heterospecific neighbors; mycorrhizal fungi can for example mediate mutualism between plants species (32). In the
265 present case, bacteria beneficial to *Dmel* host appeared detrimental to *Ds* larvae, in complete opposition with how bacteria and yeast associated to *Ds* larvae facilitate fruit use by *Dmel* (27, 33). We hence document an original example of harmful interactions between competing insects mediated by their microbiota. Humans may exploit these interactions to protect the food they grow.

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Ds is responsible for heavy crop losses throughout the globe due to the development of larvae in farmed fruit. It is tempting to exploit *Ds* oviposition avoidance to shelter fruit from *Ds* damages. However, avoidance loss as described above (Fig. 2a, 4c) means *Dmel*'s bacteria could not be directly sprayed directly in orchards. A better solution may be to use as

275 repellent the compounds produced by bacteria-inoculated *Dmel* (Fig. 4d). Obviously, it is assuming avoidance loss is based on *Ds* female colonization by repellent bacteria rather than habituation to the stimulus (9). Otherwise this repellent could only be used once per fly generation, which may be a problem as *Ds* live several months in the field (34). Identifying *D. suzukii*'s chemosensory receptors and circuitry involved in the recognition of *Dmel* cues and
280 its consequential behavioral response may also enable the design of an optimized repellent.

Society requests agricultural innovations to be sustainable. Resistance evolution has eroded the efficacy of numerous crop protection solutions. Environmentally-safe and evolution-proof alternatives, which do not lose their field efficacy over time, are therefore needed.
285 This is attainable with the repellent against *Ds* we studied here if used to prevent crop infestation. *Ds* females genetically resistant to the repellent – not to be mistaken with females that plastically lose avoidance after exposure to *Dmel* products - would deposit their eggs on fruit that bear the cues of *Dmel* microbiota. This would happen in farm treated hosts as in wild hosts. In Europe, *Ds* host range is very large, females oviposit and larvae develop in
290 about a third of the wild fruit species tested (35). In treated fruits, resistant flies would have a fitness advantage, but in untreated wild hosts, resistant females would oviposit indistinctively in both pristine fruit and fruit infested with *Dmel*. The fitness of resistant alleles would therefore be diminished by the ecological cost of larval competition, a cost not paid by susceptible alleles. The fitness of resistant genotypes relative to susceptible ones,
295 and therefore the likelihood of resistance spread, will depend on the relative frequency of a number of *Ds-Dmel* co-infestation in the wild and their effect on larval performance in a diversity of conditions, the relative importance of wild and farmed hosts for *Ds* life-cycle and the efficiency of the repellent (which varied among experiments). Other factors, such as the

evolution of *Ds* larvae's competitive ability against *Dmel* (see Text S4 Supplementary Discussion) might interfere with our goal of designing and deploying an evolution-proof repellent. Sustainability of agricultural and health solutions probably is the greatest innovative challenge evolutionary biology must tackle in order to benefit society.

Conclusions

We discovered females of the major agricultural pest *Ds* avoided depositing their eggs in sites bearing *Dmel* cues. They recognized molecules produced by the interaction of adult *Dmel* of both sexes with bacteria of their gut microbiota. *Ds* avoidance behavior revealed plastic as *Ds* inoculated with repellent bacteria stopped avoiding the cues produced by *Dmel* flies associated with the same microorganism. Avoidance behavior appeared as an adaptation by *Ds* to avoid larval competition against *Dmel* larvae, which revealed deleterious only when associated to their extra-cellular symbionts. We argue this avoidance behavior may be used to design a crop protection repellent which, in some conditions, could be evolution-proof, not giving rise to resistance emergence.

Materials and Methods

General experimental design

The study is based on a simple assay where female *D. suzukii* (*Ds*) are given the choice to lay eggs on two substrates: either a blank control or a substrate that had previously been exposed to *D. melanogaster* (*Dmel*) adults (Fig. 1). By changing the nature of the *Ds* and *Dm* flies employed, we were able to reveal the factors that govern *Dmel*'s repellence and *Ds*'s corresponding avoidance.

In most cases, a single *Ds* female was placed in a 9cm diameter plastic cylindrical box for 24h. Boxes contained two 2*2cm*2cm plastic receptacles each half-filled with oviposition substrates, generally an agar-jellified strawberry puree or a piece of strawberry inserted in blank agar. These two substrates were prepared the day before, one of them was exposed to 3 adult *Dmel* flies overnight. Because these experiments were conducted over 5 years with variable objectives, some experimental details varied among assays. Table 1 describes the experimental details and statistical analyses of each of the results reported in the article.

All flies were reared, and experiments conducted, in climatic chamber with a 13h:23°C/11h:19°C day/night cycle, an artificial dawn and dusk of 45min. Humidity was maintained constant at 75% relative humidity.

Biological material

Most experiments were carried out with our standard *Ds* population that was founded in 2013 by a few dozen individuals that emerged from blackberries harvested in Gaujac, Southern France (44.0794, 4.5781) ; and the classical *Dmel* population Oregon R, founded in 1927 and shared among laboratories since then. These fly colonies were maintained in

standard drosophila vials with banana artificial medium (see below) or 30 cm cubic cages
345 when we needed larger numbers of flies.

Additional laboratory populations of *Ds* were as follow. The Japanese population was founded from individuals captured in Matsuyama, Japan (33.8389, 132.7917) in 2015, the US population in Watsonville, California, USA (36.9144, -121.7577) in 2014, and the Chinese population in Shiping, China (23.7048, 102.5004) in 2015. The *D. simulans* population tested
350 was founded from individuals captured in 2015 in Lyon, France (45.7835, 4.8791). All populations were initially composed of a few individuals and experienced repeated population bottlenecks during maintenance. They were thus largely inbred at the time of testing in 2017.

355 Wild *Ds* were captured during summer 2016 in two localities 10km apart near Montpellier, Southern France (43.6816, 3.8776), and tested about a week after capture, once they started laying eggs in the laboratory. Wild *Dmel* were also captured near Montpellier. For the experiment reported in Fig. 3b, *Dmel* flies were captured in several instances. Flies from a first group were reared in the laboratory and their offspring (*i.e.* F1) tested along with
360 freshly-captured flies (*i.e.* F0). All wild flies were captured using custom-designed traps based on c.300 mL plastic cups, covered with cling-film, pierced on the sides for fly entry and containing an attractant (a mix water, vinegar, wine and sugar) separated from the flies by netting. The netting prevented fly drowning but allowed occasional access to the attractant as cups were readily shaken by wind or operators, which provoked netting soaking with
365 liquids. Traps were checked daily and usually contained various fly species, including *Dmel* and *Ds*.

Recipes for rearing and oviposition media

370 Laboratory flies were reared on custom banana medium (1.2 L water, 280 g frozen organic banana, 74 g glucose, 74 g inactivated baker's yeast, 12 g agar, 6 g paraben in 30 mL ethanol). The Chinese *Ds* population was reared in carrot medium (1.2 L water, 45 g carrot powder, 45 g glucose, 27 g inactivated baker's yeast, 18 g corn meal, 13.5 g agar, 6 g paraben in 30 mL ethanol and 4 mL propionic acid).

375 In most cases, oviposition was assayed on strawberry puree (200 g frozen strawberry, 400 mL water, 6 g agar, 37 g glucose, 4 g paraben in 15 mL ethanol). In several instances (Table 1), we used jellified grape juice (100 mL commercial grape juice, 100 mL water, 12 g glucose, 2 g agar). Oviposition was also tested on pieces of strawberry inserted in jellified water (100 mL water, 1 g agar), they were first bleached (0.6% bleach during 5 min) to remove

380 contaminants.

Axenics, mono-associated flies and microbiological work

Axenic flies were produced following a protocol derived from (36). Briefly, *Drosophila* eggs

385 were collected on grape-juice medium (see previous recipes section) before being bleached and rinsed twice (1.2% sodium hypochlorite). Eggs were then transferred to 50 mL centrifugation vials with 10 mL autoclaved banana medium (see recipes section) which lids were either incompletely screwed of harboured breathing membranes. All manipulations were conducted under a laminar flow hood. With care, it is possible to transfer freshly

390 emerged adults to new vials aseptically and therefore maintain the population microbe-free for several generations.

To produce mono-associated (i.e. gnotobiotic) adult flies, vials containing axenic flies were inoculated with suspensions (i.e. >10^5 cells) of the relevant bacterium at least 4 days before experiment onset. Adults used for an experiment were discarded to avoid contamination of
395 subsequent assays.

Larval competition between D. suzukii and D. melanogaster in fruit

This assay aimed at testing whether the development of *Ds* larvae was affected by the
400 presence of *Dmel* larvae and their associated microbiota. We took great care of reproducing field-like conditions (i.e. in-fruit interactions) as competition costs notoriously depend on ecological conditions (e.g. 37) and the effects of *Drosophila* bacterial symbionts on larval development change with medium richness (e.g. 38). To this end we developed a new infestation protocol for *Ds* where we mimicked natural oviposition. We manually pierced the
405 skin of grape berries with fine needles, making a hole close in size as those *Ds* females do with their ovipositor (39). Each hole was first inoculated with a wild strain of the yeast *Hanseniaspora meyeri* isolated from wild *Ds* adults and received a single *Ds* egg (Fig. S3). There were 6 holes per berry. Each of these holes also received 0, 1 or 5 *Dmel* eggs. The larger ratio of *Dmel* to *Ds* eggs was chosen as it reflects relative infestation intensity
410 observed in grapes collected in the field (e.g. 27). In half the cases, deposited *Dmel* eggs had been made axenic by bleaching (see previous section on the production of axenic flies). In the treatments without *Dmel* eggs but with its microbiota, pierced berries were exposed to 10 *Dmel* males for 24h prior to *Ds* egg deposition. All grape berries were incubated in individual plastic vials until adult flies emerged. This assay comprised 25-30 individual berries
415 per treatment (50 replicates for the control treatment with *Ds* eggs and no *Dmel* microbiota)

spread over 8 temporal blocks. For brevity, we only present here the results for *Ds* survival until emergence, other results – which are largely congruent with those in this manuscript – will be published separately.

420 ***Statistical analyses***

In all reported experiments, but the one on larval competition (Fig. 5), *Ds* females deposited their eggs on either treated or untreated oviposition substrates. Egg counts on each type of medium were therefore not independent as they were produced by the same females.
425 Besides, total number of eggs varied among females and experiments and largely followed a Poisson distribution, which prevented the use of traditional linear models that assume normal distributions of the residuals. We therefore used a simple, robust statistical approach to analysing the proportion of eggs deposited on treated and untreated site: a non-parametric, one-tailed Wilcoxon signed rank test that took into account data pairing and was
430 compatible with its distribution. We however noticed that paired t-tests, which assume data follows a normal distribution, provided similar results. Because of the plasticity of the avoidance behaviour, all experiments included a positive control - usually the response of standard *Ds* to laboratory *Dmel* flies. This ensured lack of avoidance in an experiment was not due to unidentified factors or inappropriate conditions. Note several of our most
435 important results were repeatedly observed in distinct experiments ; compare, for example, loss of avoidance in Fig. 2a and Fig. 4c, effect of axenic *Dmel* in Fig. 4a and Fig. 4b, restauration of *Dmel* repellency by *Lactobacillus brevis* inoculation in Fig. 4b and Fig. 4c.

Results from the larval competition assay were analysed using a linear mixed-model with the REML method. Numbers of *Ds* adult that emerged from each fruit were Log(x+1)-transformed and complied with tests assumptions. This model contained discreet, fixed terms describing the number of *Dmel* eggs deposited, whether *Dmel* microbiota was present and their interaction. We also included the log-transformed number of *Dmel* adults that emerged from the fruit as a fixed, continuous factor. This term was necessary as the bleaching of *Dmel* eggs provokes some mortality; it enabled controlling for the actual numbers of live *D. melanogaster* larvae that competed with *Ds* larvae. The model also included a block term (treated as random). Differences among treatments were tested with independent contrasts.

All analyses were carried out with the software JMP 14.0 (SAS Institute Inc. 2018). Throughout the manuscript, stars in figures indicate significance of one-tailed statistical tests: * $p<0.05$; ** $p<0.01$; ***$p<0.001$; n.s. $p>0.05$.

All data is available on the Zenodo platform under the reference: 10.5281/zenodo.3970737.

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Figures and Tables

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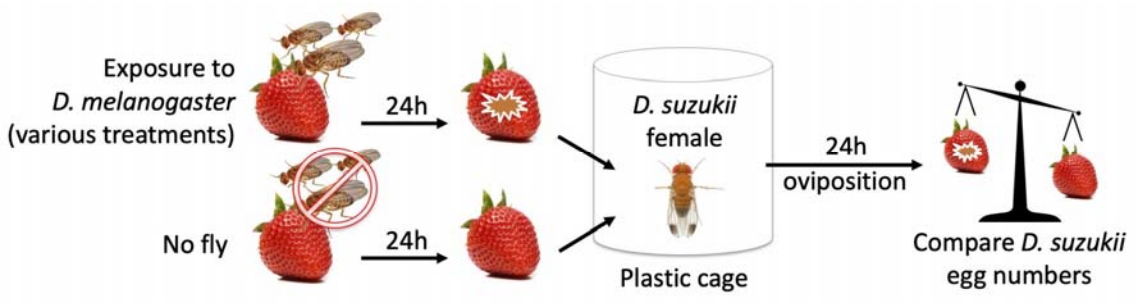
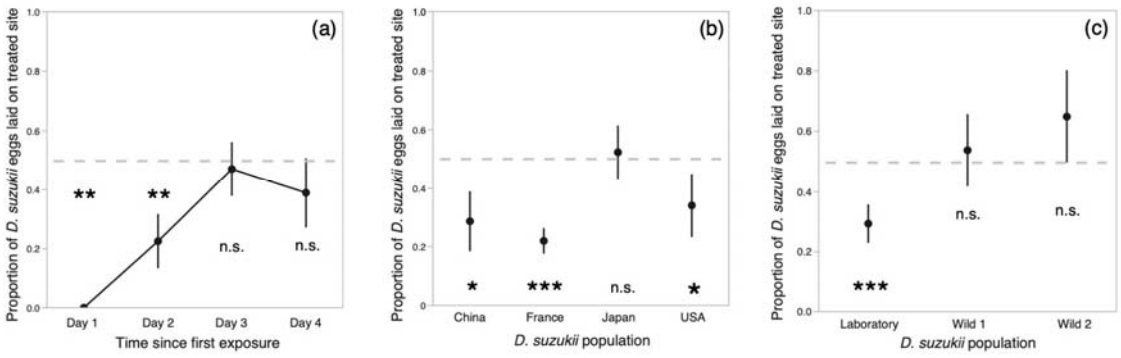


Figure 1: Schematic drawing of the experimental procedure for testing *D. suzukii* oviposition

585 avoidance of egg-laying sites previously exposed to *D. melanogaster*. Details of each experiment, among which origin, sex and numbers of *D. suzukii* and *D. melanogaster* flies, cage size and oviposition substrate, are described in Table 1 of the Materials and Methods.



590 Figure 2: oviposition avoidance of *D. suzukii* females for egg-laying site previously exposed to *D. melanogaster*. Values significantly below 0.5 indicate *D. suzukii* preference for sites unexposed to *D. melanogaster*. Repeated tests of the same females (a) show plastic avoidance loss. *D. suzukii* populations from different geographical origins (b) exhibit variable avoidance. Trap-captured, wild *D. suzukii* females (c) did not avoid oviposition on *D. melanogaster* exposed substrates. Symbols

595 indicate means and error-bars standard errors. Significant deviation from equal number of eggs on sites exposed to *D. melanogaster*, or control sites, were produced by one-tailed Wilcoxon signed rank tests ; * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$.

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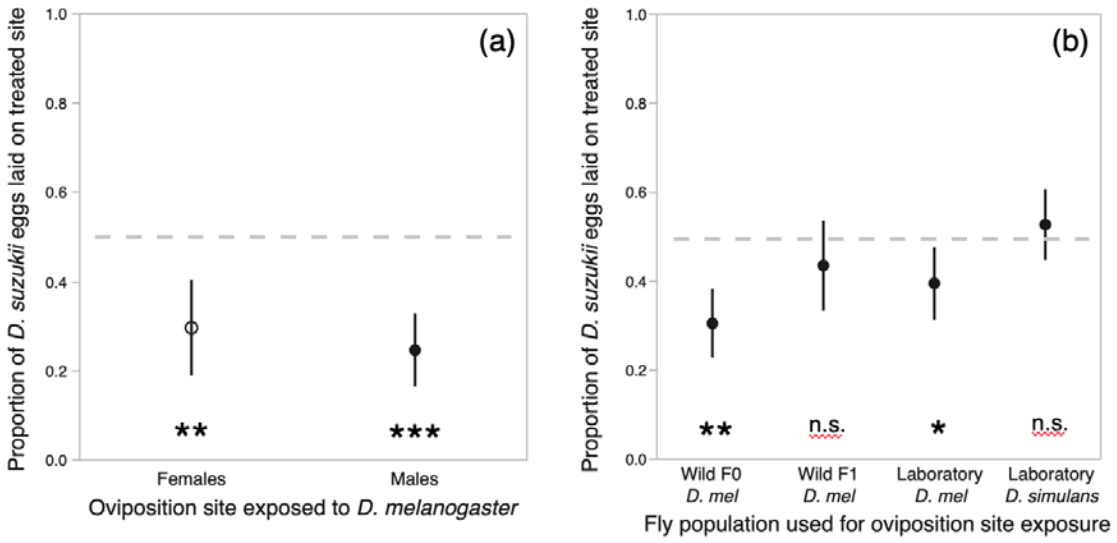

Figure 3: oviposition preferences of *D. suzukii* females for egg-laying site previously exposed to different *Drosophila* flies. *D. melanogaster* males, like females, (a) induce repellency. Trap-captured, wild *D. melanogaster* flies (F0 in b) induced repellency, however this property was not induced by

605 laboratory-reared offspring from wild-caught flies (F1 in b) nor by *D. simulans* . Symbols indicate means and error-bars standard errors. Statistical tests produced by Wilcoxon signed rank tests; * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$.

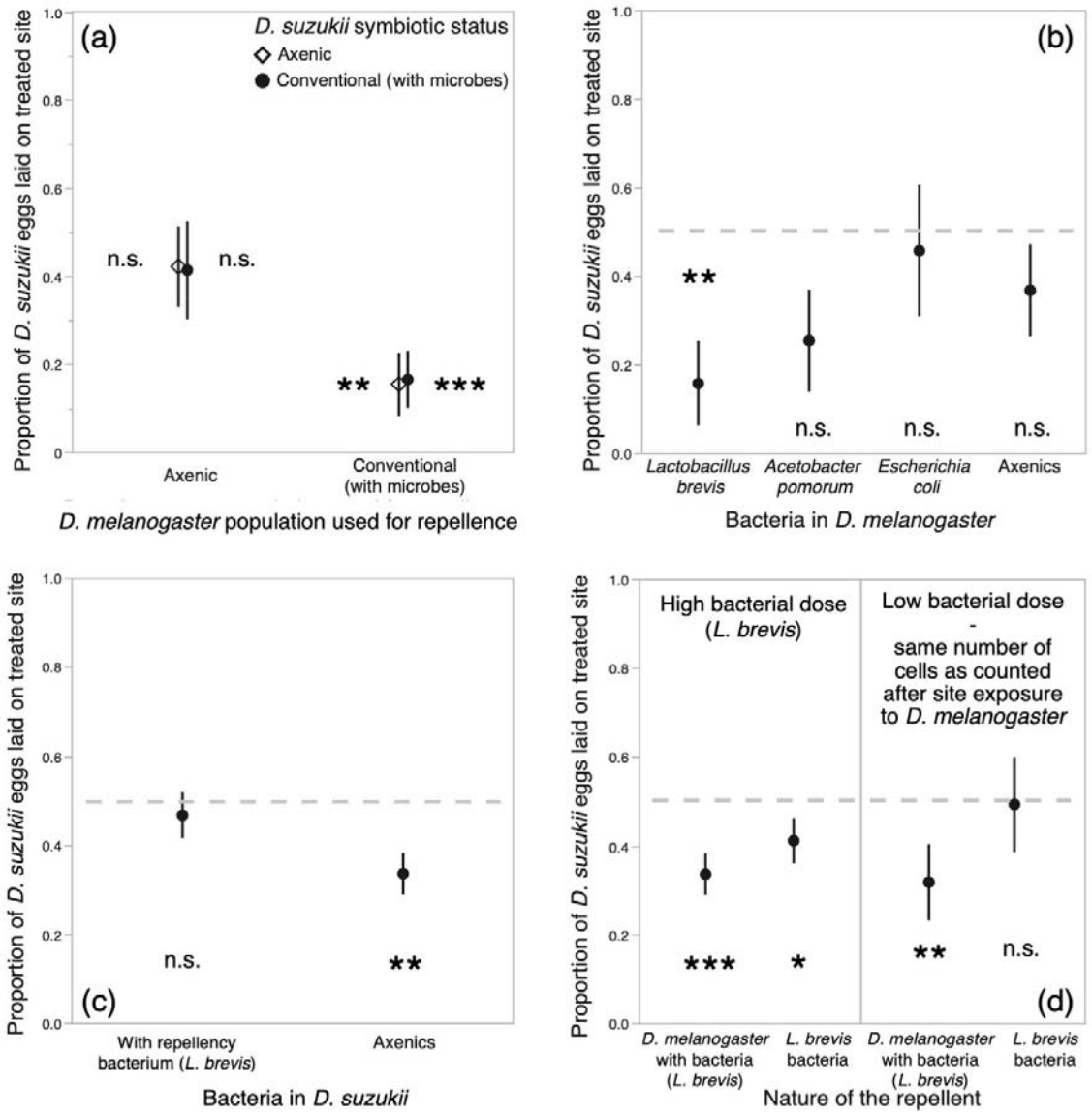

Figure 4 : investigation of the role of extra-cellular symbionts on *D. melanogaster* repellence and *D. suzukii* oviposition avoidance. Axeny, the removal of extra-cellular microorganisms, (a) had different effects on *D. melanogaster* and *D. suzukii*. Oviposition sites exposed to axenic *D. melanogaster* were not avoided by *D. suzukii*, showing the implication of symbionts in *D. melanogaster* for repellence. By contrast, axenic *D. suzukii* behaved like conventional flies; *D. suzukii* microorganisms were therefore not mandatory for perceiving the repellent. Test of candidate bacteria in association with *D. melanogaster* (b) revealed the bacterium *Lactobacillus brevis* can restore repellence in formerly axenic flies (note the axenic and *Acetobacter pomorum* treatments were marginally non-significant,

p= 0.071 and p= 0.075, respectively). We hypothesized *D. suzukii* avoidance loss was due to their colonisation with *D. melanogaster* symbionts. As expected, *D. suzukii* females experimentally
620 associated with the bacterium *L. brevis* (c) did not avoid oviposition on sites exposed to *L. brevis*-associated *D. melanogaster* . Direct inoculation of medium with *L. brevis* cells (d) in large numbers or at a dose similar to that naturally shed by *D. melanogaster* (i.e. 1,000,00 *vs* 5,000) produced different results. The low, natural dose of deposited bacteria failed to elicit avoidance, suggesting *D. melanogaster* repellence is largely due to the production of unidentified molecules when in
625 symbiosis. Symbols indicate means and error-bars standard errors. Statistical tests produced by Wilcoxon signed rank tests; * for p< 0.05; ** for p< 0.01; *** for p< 0.001.

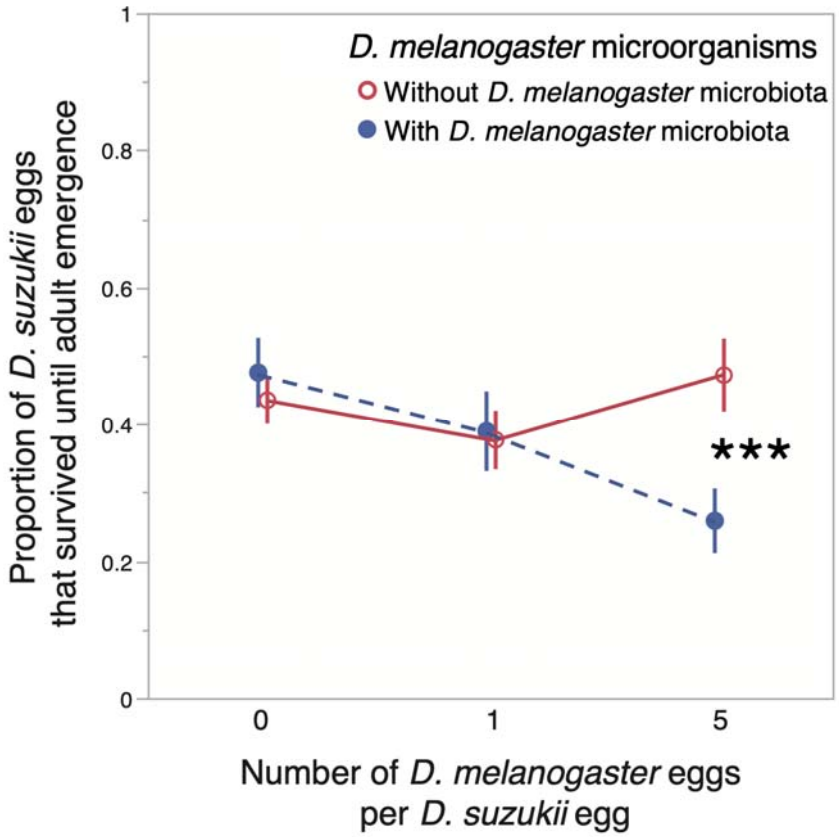

Figure 5: effect of *D. melanogaster* larvae and their associated microbiota on the development of *D. suzukii* eggs until adult emergence. Eggs were individually deposited in grape berries where we mimicked natural oviposition by *Drosophila* females and field-like conditions. The greater ratio of D. melanogaster to D. suzukii egg follows relative infestation intensities observed in the field. The statistical interaction between number of *D. melanogaster* eggs and the presence or absence of their microbe was significant ($F_{2, 172}$= 6.46; p= 0.002). Independent contrasts indicate a significant difference between the treatments with and without *D. melanogaster* microbes at high *D. melanogaster* density ($F_{1, 174}$= 15.6; p= 0.0001). Symbols indicate means; error bars indicate standard-errors; *** for p< 0.001.

Table 1: details of the experiments presented in the main text and statistical results.

Experiment question and figure presenting the results	**Method**	**Number of *Ds* females in assay; type of container**	***Ds* particulars**	***Dmel* particulars**	**Oviposition substrate**	**Replication and raw statistical results** Reported number of replicates excludes the frequent cases where no *Ds* eggs were deposited during the experiment	**Additional comments**
- Do *Ds* females maintain avoidance behaviour over time? - Fig. 2a	We repeatedly assayed the behaviour of the same females over 4 consecutive days renewing the oviposition substrates daily.	- Initially 10 females, some mortality occurred during the 4 days of the experiment - 30cm diameter cylinders	Standard laboratory population	Standard Oregon R laboratory population	- Jellified grape juice - 5cm petri-dishes	We followed 9 cages throughout the 4 days. **Wilcoxon signed rank tests, one-tailed:** Day 1: S= 21; p= 0.008 Day 2: S= 20.5; p= 0.006 Day 3: S= 6; p= 0.28 Day 4: S= 9; p= 0.18	On days 1 and 3 *Ds* females were given the choice between *Dme*-exposed and control substrates. On day 2 and 4 they were given the choice between *Dmel*- and *Ds*-exposed substrates.
- Is avoidance behaviour present in various *Ds* populations? - Fig. 2b	We assayed avoidance behaviour in 4 laboratory populations of different regional origin	- 1 female per assay - 9 cm diameter cylinders	Standard laboratory population and 3 populations founded from individuals captured in China, the USA and Japan	Standard Oregon R laboratory population	- strawberry puree - 2*2 cm cubic receptacles	**Wilcoxon signed rank tests, one-tailed:** China: n= 14, S= 32.5; p= 0.019 France: n= 57, S= 557; p< 0.0001 Japan: n= 27, S= -3.5; p=0.53 USA: n= 16, S= 42; p=0.014	Details of the tested *Ds* populations are available in the biological material section
- Do wild *Ds* females avoid *Dmel* exposed media? - Fig. 2c	We captured wild adult flies and assayed their behaviour in the laboratory.	- 1 female per assay - 9cm diameter cylinders	Laboratory and wild flies	Wild *Dmel*	- strawberry puree - 2*2cm cubic receptacles	**Wilcoxon signed rank tests, one-tailed:** Laboratory: n= 19, S= 74; p= 0.0008 Wild 1: n= 12, S= 6.5; p= 0.28 Wild 2: n=8, S= -15; p= 0.98	Wild *Ds* females were trap-captured in two localities near Montpellier, France. Traps usually contained a diversity of fly species, including *Dmel*. *Ds* females were kept in the laboratory for several days before they started laying eggs and could be assayed.
- Are male *Dmel* repellent? - Fig. 3a	Oviposition substrates exposed to either males or females *Dmel*.	- 1 female per assay - 9cm diameter cylinders	Standard laboratory population	Standard Oregon R laboratory population	- jellified grape - 2*2cm cubic receptacles	**Wilcoxon signed rank tests, one-tailed:** Female *Dmel*: n= 16, S= 54; p= 0.0013 Male *Dmel*: n= 21, S= 83.5; p= 0.0007	When *Ds* oviposition substrates had been exposed to *Dmel* females that oviposited too, eggs from each species could be discriminated thanks to the elongated respiratory tubes that are specific to *Ds*.
- Are wild *Dmel* and	We captured wild adult	- 1 female per assay	Standard laboratory	Laboratory and	- jellified grape	**Wilcoxon signed rank**	Details of the tested *Dmel*

laboratory *D. simulans* repellent ? - Fig. 3b	*Dmel* and tested their repellency. We also tested the repellency of F1 offspring from wild-*Dmel*. A *D. simulans* population laboratory was included in the assay	- 9cm diameter cylinders	population	wild *Dmel* ; offspring of- wild *Dmel* flies (i.e. F1) ; laboratory *D. simulans* population.	juice - 2*2cm cubic receptacles	**tests, one-tailed:** Wild F0: n= 17, S= 57.5; p= 0.0032 Wild F1: n= 16, S= 6.5; p= 0.40 Laboratory: n=23, S= 62.5; p= 0.027 *D. simulans*: n= 19, S= 7; p= 0.40	populations are available in the biological material section
- Is *Dmel* repellence exerted at a distance? - Fig. S2	We tested whether medium surrounding medium exposed to *Dmel* received less *Ds* eggs than medium surrounding pristine medium.	- 1 female per assay - 20cm cubic netting cages	Standard laboratory population	Standard Oregon R laboratory population	- strawberry puree - 2*2cm cubic receptacles and 12cm square petri dishes	**Wilcoxon signed rank tests, one-tailed:** Exposed area: n= 29, S= 101; p= 0.013 Peripheral area: n= 29, S= -14; p= 0.61	Details of the protocol are described in the supplementary materials
- Do axenic *Ds* and *Dmel* flies maintain avoidance and repellence? - Fig. 4a	We produced axenic *Ds* and *Dmel*, which we compared to conventional flies. They were tested in a full-factorial set-up.	- 1 female per assay - 9cm diameter cylinders	Standard laboratory population; conventional and axenic	Standard Oregon R laboratory population; conventional and axenic	- strawberry puree - 2*2cm cubic receptacles	**Wilcoxon signed rank tests, one-tailed:** Conventional *Dmel* and conventional *Ds*: n= 27, S= 133; p= 0.0001 Conventional *Dmel* and axenic *Ds*: n= 17, S= 60.5; p= 0.002 Axenic *Dmel* and conventional *Ds*: n= 16, S= 19.5; p= 0.11 Axenic *Dmel* and axenic *Ds*: n= 16, S= 8.5; p= 0.39	Details for the production of axenic flies are described in the appropriate section
- Test of bacterial candidates possibly involved in *Dmel* repellence - Fig. 4b	We mono-associated axenic *Dmel* flies (i.e. made gnotobiotic flies) with two of their most important bacterial gut reported in the literature, *Lactobacillus brevis* and *Acetobacter pomorum*. We also tested *Dmel* associated with *Escherichia coli*.	- 1 female per assay - 9cm diameter cylinders	Standard laboratory population	Axenic and mono-associated Oregon R flies	- Pieces of bleached strawberries maintained in agar jelly - 2*2cm cubic receptacles	**Wilcoxon signed rank tests, one-tailed:** *L. brevis*: n= 14, S= 40.5; p= 0.0043 *A. pomorum*: n= 13, S= 22; p= 0.0745 *E. coli*: n=11, S= 10.5; p= 0.18 Axenics: n= 17, S= 31; p= 0.071	Details for the production of axenic and mono-associated flies are described in the appropriate section
- Do *Ds* females that bear repellence bacteria still avoid oviposition in sites exposed to *Dmel* associated with the same bacteria? - Fig. 4c	We compared the behaviour of axenic *Ds* females to that of conspecific associated with the same bacterium as in *Dmel*	- 1 female per assay - 9cm diameter cylinders	Axenic and mono-associated with *Lactobacillus brevis*	Oregon R flies mono-associated to *Lactobacillus brevis*	- strawberry puree - 2*2cm cubic receptacles	**Wilcoxon signed rank tests, one-tailed:** Axenics: n= 29, S= 147; p= 0.0003 Associated to L. brevis: n= 28, S= 54; p= 0.11	Details for the production of axenic and mono-associated flies are described in the appropriate section

- Are bacteria alone sufficient to elicit *Ds* avoidance? - Fig. 4d	We deposited cells of the bacterium *L. brevis* on oviposition medium, let it rest overnight as for *Dmel* exposure, and tested whether it elicited *Ds* avoidance. This assay was repeated with high and low cell numbers, the latter corresponding to the number of cells retrieved on medium surface after *Dmel* exposure in the standard conditions of our experiments.	- 1 female per assay - 9cm diameter cylinders	Axenics	mono-associated Oregon R flies (i.e. bacteria in *Dmel*) and pure bacteria from liquid culture	- strawberry puree - 2*2cm cubic receptacles	**HIGH bacterial dose (1,000,000):** **Wilcoxon signed rank tests, one-tailed:** *Dmel* exposure: n= 29, S= 147; p= 0.0003 Bacteria only: n= 29, S= 103; p= 0.011 **LOW bacterial dose (5,000):** **Wilcoxon signed rank tests, one-tailed:** *Dmel* exposure: n= 22, S= 82.5; p= 0.002 Bacteria only: n= 11, S= 2.5; p= 0.58	Details for the production of axenic, mono-associated flies and purified bacteria are described in the appropriate section
- Does the microbiota of *Dmel* larvae affect their influence on *Ds* development? - Fig. 5	Details of this experiment are reported in a separate Material and Methods section					**REML mixed-model:** Number Dmel eggs per Ds egg ; $F_{2,169}$= 4.83; p= 0.009 Dmel axenic or not; $F_{1,162}$= 0.41; p= 0.52 Number of Dmel eggs * axenic or not; $F_{2,172}$= 6.46; p= 0.002 Number of emerging Dmel adults; $F_{1,174}$= 7.74; p= 0.006	Results from the statistical analyses were similar also when neglecting the actual number of *D. melanogaster* individuals that survived and emerged

Supplementary Materials

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S1. Do *Ds* female avoid oviposition in sites with eggs from conspecifics?

In a preliminary assay, similar to that of Shaw *et al.* (1) we tested whether *D. suzukii* (*Ds*)

650 females avoided oviposition on media containing eggs from conspecific. These eggs were naturally deposited by females on artificial medium and their numbers ranged between 19 to 147. The experiment was conducted in 30 cm diameter cylinder cages with 10 females over two successive days keeping the same females.

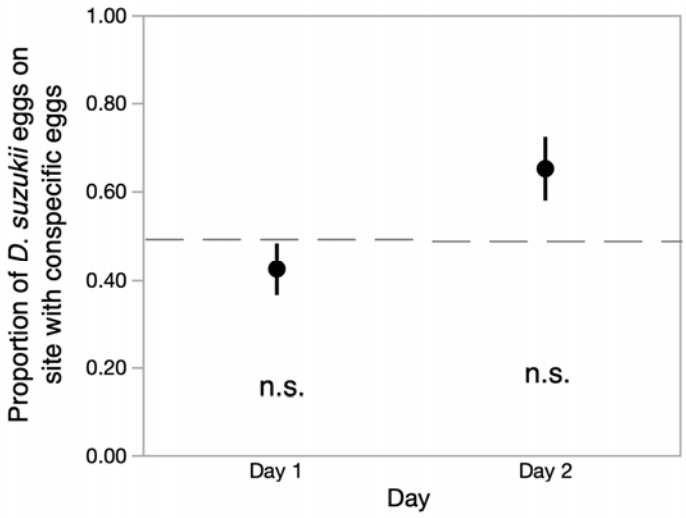

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Fig. S1: presence of *D. suzukii* eggs did not elicit *D. suzukii* oviposition avoidance. Wilcoxon signed rank tests were not significant on either day. Symbols indicate means and error-bars standard errors.

660 **S2. Short-range repellence by *D. melanogaster***

In order to test whether *D. melanogaster* (*Dmel*) repellency could proceed at a distance, for example through odorant volatiles, we designed an assay based on the one of (2). We used the same 2-cm cubic receptacles as for most oviposition assays, which we exposed to *Dmel*
665 for 24h (control media were not exposed to Dmel). These receptacles were placed with the aperture on the side on 12cm square petri dishes containing jellified grape juice within a 20 cm cubic netted cage. The assay was then conducted as usual with single *Ds* females from our standard population. After 24h, we counted the number of eggs on the medium in the small receptacle that contained exposed (or unexposed) strawberry puree as well as on the
670 surrounding medium.

As expected, exposed media received less *Ds* eggs than unexposed ones. However, the effect did not extend to the surrounding substrates (Fig. S2). This shows that repellency does not rely on long-range odors and suggests gustation and direct contact may be required by *Ds* females to assess *Dmel* cues.

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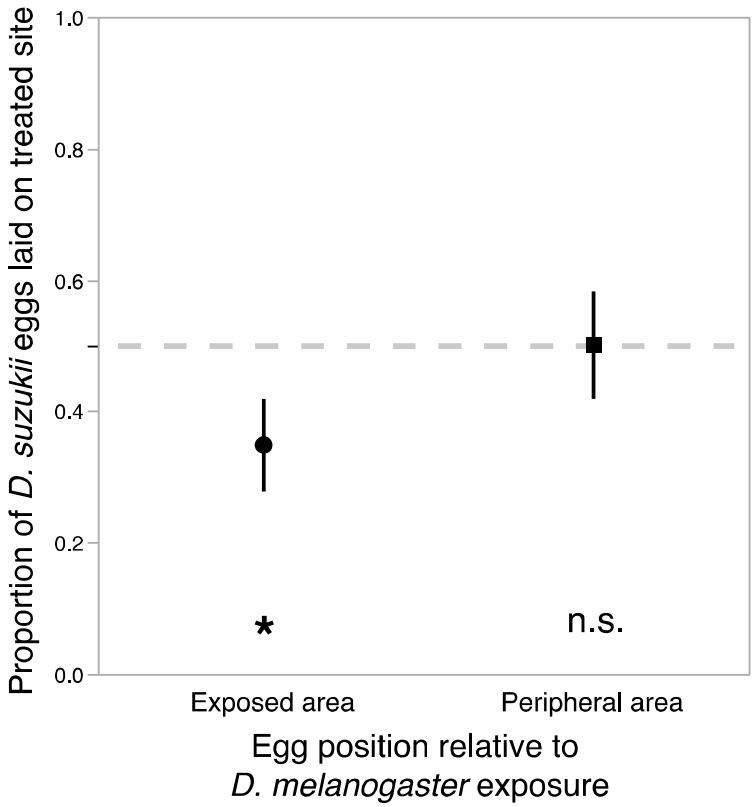
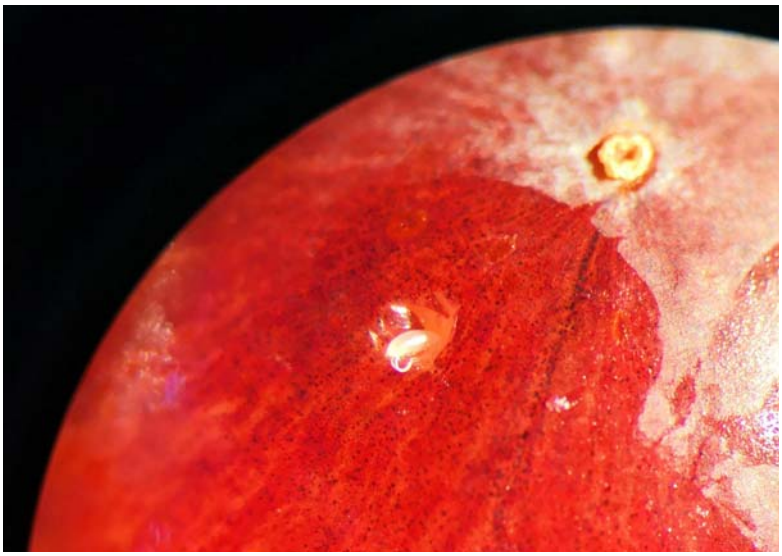


Fig. S2: fresh medium (right) placed close to medium previously exposed to *D. melanogaster* (left) is not avoided by egg-laying *D. suzukii* females. Symbols indicate means and error-bars standard errors.

680 Statistical tests produced by Wilcoxon signed rank tests; * for $p < 0.05$.

S3. Protocol for within-fruit competition between larvae



685 Fig. S3: A single *D. suzukii* egg manually deposited in an artificial oviposition hole. In the experiment reported here, fruit bore 6 such eggs. In some treatments, 1 or 5 *D. melanogaster* eggs were also deposited in the hole. In half of these treatments, *D. melanogaster* eggs had been made axenics using bleach baths.

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S4. Supplementary discussion

A phylogenetic perspective on the *Drosophila* genus shows few species oviposit in undamaged, ripening fruit (3). It is therefore likely *Ds* ancestors evolved the ability to exploit ripening fruits in order to alleviate competition with other Drosophilids. *Dmel* arrived in Asia less than 60 000 years ago, that is long after *Ds* speciation (4). The larval niche of *Ds*, and possibly female oviposition preferences, hence probably evolved in response to other species of Drosophilids. In our experiments *Ds* did not avoid *D. simulans* cues. It is nonetheless plausible *Ds* females avoid cues produced by other Drosophilid species or populations, in particular those from the region it originates, or that this depends on the symbiotic status of these flies.

It is often argued costs of resistance are key factors that prevent resistance to natural or human-made stressors (e.g. parasites or chemical pesticides) (5, 6). In numerous systems, resistance costs have eroded over time due to the emergence of compensatory mutations or less-costly resistance solutions (7). Can *Ds* become resistant to the repellent without suffering from larval competition with *Dmel*? It is unlikely since *Ds* has been in contact with *Dmel* for several thousand years in Asia (4) without evolving such ability. Costs of resistance to natural stressors - against which the target species has been exposed for a long time without evolving costless resistance - may therefore maintain over time. A situation different from resistance to xenobiotics, such as chemical insecticides, which the target species has never encountered in the past. In other words, an old stressor the target species has not managed to overcome may reveal more evolution-proof than a new stressor untested by evolution.

715

References for Supplementary Materials

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